EVIDENCE AGAINST TUBULIN–ACTIN HOMOLOGY

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1. Introduction

The microtubule protein, tubulin, from brain, blood platelets, cilia and flagella and the microfilament protein, actin, from muscle and cytoplasmic sources all have similar amino acid compositions [1,2] and the involvement of the polymeric forms of these two subunit proteins in a variety of cellular motile activities has raised the question of the possible presence of regions of homology in their structure. This has become a topic of some considerable controversy. Puszkin and her colleagues [3,4] reported that colchicine-binding proteins (or presumptive tubulins) isolated from brain and blood platelets enhanced over 20-fold the Mg^{2+}-dependent ATPase activity of muscle myosin, a property generally regarded as specific for actin [5]. Stephens, however, in a fingerprint study of tryptic and chymotryptic peptides of adductor muscle actin and ciliary tubulin from the scallop *Pecten irradians* clearly demonstrated that these two proteins had very dissimilar two-dimensional peptide patterns [6]. In another report Mohri and Shimomura [7] compared the properties of tubulin and actin isolated respectively from sea-urchin sperm flagella and sea-urchin latern muscle and showed that although both proteins formed superprecipitates with rabbit skeletal muscle myosin in the presence of ATP, only the latern muscle actin increased the Mg^{2+}-dependent ATPase activity of muscle myosin at low ionic strength. Furthermore, only the synthetic actomyosin and not the tubulin–myosin mixture showed an ATP-inducible viscosity decrease under high ionic strength conditions. In a more recent study however, Alicea and Renaud [8] examined the effect of *Tetrahymena* ciliary tubulin on the Mg^{2+}-dependent ATPase of chicken breast muscle myosin and recorded an almost 4-fold increase in Mg^{2+}-ATPase activity. They concluded that this was strong evidence for actin tubulin homology.

In our studies [9,10] of the properties of actins and tubulins isolated from pig platelets and mammalian brains we showed that although there are some similarities in certain properties the two subunit proteins differ significantly in their molecular weights, drug binding affinities, subcellular localisation and the polymeric structures which they form. Although the amino acid compositions of platelet and brain tubulin are virtually identical, they differ significantly from those of platelet and muscle actins in at least 6 residues [2]. Furthermore, fluorescently-labelled anti-actin and anti-tubulin antibodies stained morphologically quite different structures in bovine lens epithelial cells [10].

In the light of these findings and the controversy in the literature, we felt it pertinent to re-examine the homology question, and in this paper we present experimental results which we consider do not support the concept of tubulin–actin homology. The studies have been based largely upon actin–myosin and tubulin–myosin combination experiments but since it has also recently been shown that G-actin inhibits the enzyme DNase I and forms a stoichiometric complex with it [11], we have also compared the effect of muscle actin and brain tubulins on DNase I activity.

2. Materials and methods

2.1. *Materials*

Mes*, EGTA, GTP (Type 11-S), DNA and DNase I were obtained from the Sigma Chemical Co. Ltd., and all other chemicals were of analytical grade where possible.

Random mated Wistar-derived adult rats were used and fresh calf brains were obtained from animals killed at a local abattoir.

2.2. *Protein preparations*

Rat and calf brain tubulins were prepared by the in vitro polymerisation procedure of Shelanski et al. [12], with two complete cycles of polymerisation/depolymerisation. Calf brain tubulin was further purified by passing the cold depolymerised protein through an Ultrogel AcA34 column to obtain tubulin free from high-molecular weight proteins and other minor contaminating proteins.

Rabbit skeletal muscle actin was extracted from a dried acetone powder of muscle by the method of Bailin and Bárány [13] and purified by two cycles of polymerisation/depolymerisation. Rabbit skeletal muscle myosin was isolated by the procedure of Stone [14] after Perry [15].

2.3. *Protein determinations*

All protein values except those for myosin, were determined by the procedure of Lowry et al. [16] using bovine serum albumin as a standard. The concentration of myosin in 0.6 M KCl was determined using the microtannin turbidity test for protein devised by Katzenellenbogen and Dobryszycka [17].

2.4. *SDS-Polyacrylamide gel electrophoresis*

The proteins were denatured by making to a final concentration of 4% SDS, 8 M urea and 0.1 M β-mercaptoethanol and placing in a boiling water bath for 5 min. SDS-polyacrylamide stick gels (5% and 7.5% w/v and size 9 × 0.5 cm) were run in a buffer system of 0.4 M boric acid adjusted with Tris (approx. 0.1 M) to pH 7.0 containing 0.1% sodium dodecyl-sulphate. Bromophenol blue was used as tracking dye with a current density of 2–3 mA/gel. The gels were stained overnight in 0.25% Coomassie Brilliant Blue R in methanol–acetic acid–water (45:10:45 by volume) and destained in methanol–acetic acid–water (1:1:8 by volume).

For molecular weight determinations a series of standard proteins (myosin heavy chain, phosphorylase a, catalase, ovalbumin, trypsin and ribonuclease) were used for mobility comparison.

2.5. *Determinations of Mg^{2+}-dependent ATPase*

Samples, (total assay volume 2.0 ml), were incubated at 37°C for 1 h and the liberated inorganic phosphate determined by the method of Martin and Doty [18]. The constituents of each assay mixture are given in the results section. Appropriate non-enzyme and no substrate controls were included with each set of assays.

The Tris salt of ATP was used as a substrate for the ATPase assay, carried out at pH 7.4 in Tris–HCl buffer. For experiments at other pH values the sodium salt of ATP was used and a special 'cocktail buffer' was prepared as follows: 90 ml of stock buffer (3 mM sodium acetate, 120 mM sodium succinate, 120 mM sodium maleate and 120 mM Tris) was adjusted with HCl to the required pH and made to 100 ml with distilled water.

2.6. *DNase I assay*

The DNase I assays were performed exactly as described by Lindberg [19] using their preparative procedures for the DNA substrate and the DNase I. Absorbance readings were made on a Unicam SP 1800 spectrophotometer.

3. Results

3.1. *Purity of protein samples*

All rabbit skeletal muscle myosin preparations showed in SDS gels a prominant band of low mobility, i.e. the myosin heavy chain, of molecular weight 200 000 (fig.1a). No actin could be detected in the myosin preparations as judged by SDS-polyacrylamide gel electrophoresis with overloaded gels. Rabbit skeletal muscle actin preparations gave only a single band on SDS gels, with a mobility corresponding to a

*Abbreviations: MES (2[*N*-Morpholino]ethane sulphonic acid); EGTA, ethyleneglycol-bis-(β-amino-ethyl ether) *N,N*-tetra-acetic acid; SDS, sodium dodecyl sulphate.

molecular weight of 42–44 000 (fig.1b). Rat and calf brain tubulin samples, prepared by two cycles of in vitro polymerisation, showed a protein band on SDS-polyacrylamide gels with a mol. wt. of 55 000 together with two or possibly three proteins of higher molecular weight (i.e. > 200 000). These high-molecular weight proteins accounted for between 9% and 15% of the total protein in the tubulin preparations. No band of mobility corresponding to actin was observed in any tubulin preparation (fig.1c). When actin and tubulin were co-electrophoresed on the same gel the two proteins migrated as separate species, the actin having a greater mobility than the tubulin (fig.1d).

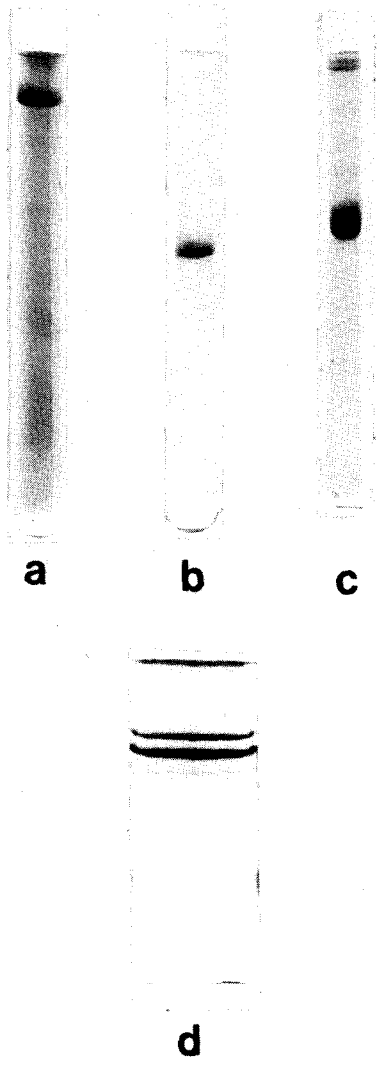

Fig.1. SDS-polyacrylamide gel electrophoresis (7.5% gels) of: (a) A rabbit skeletal muscle myosin preparation. (b) A rabbit skeletal muscle actin preparation. (c) A rat brain tubulin preparation obtained after two cycles of polymerisation/depolymerisation. (d) Co-electrophoresis of rabbit skeletal muscle actin and a rat brain tubulin preparation.

Calf brain tubulin of a higher degree of purity was prepared by applying a cold depolymerised tubulin preparation (obtained after two cycles of polymerisation/depolymerisation), to a AcA34 Ultrogel column. Two protein peaks were eluted from the column and analysis by SDS-polyacrylamide gel electrophoresis showed that the first peak, which eluted in the void volume, (Peak 1, fig.2) contained the high-molecular weight proteins and a small amount of tubulin. The second peak (fig.2, Peak II) contained only tubulin, the molecular weight of which was estimated to be 55 000 by SDS-polyacrylamide gel electrophoresis.

3.2. *The effect of rabbit skeletal muscle actin and mammalian brain tubulins on the Mg^{2+}-dependent ATPase of rabbit skeletal muscle myosin*

Figure 3 shows the effect of adding increasing quantities (0–200 μg) of rabbit skeletal muscle actin or rat brain tubulin (0–200 μg) to a fixed amount (20 μg) of rabbit skeletal muscle myosin. The Mg^{2+}-dependent ATPase of each mixture was measured after 1 h incubation at 37°C and the specific activities expressed in terms of the myosin concentration in the mixtures.

Rabbit skeletal muscle actin significantly increased the Mg^{2+}-dependent ATPase activity of the myosin. At an actin–myosin ratio of 10:1 (w/w) the ATPase activity was over 15 times greater than the activity measured in the absence of actin.

When rat brain tubulin, which had been prepared by two cycles of in vitro polymerisation, was added to the myosin, the Mg^{2+}-dependent ATPase activity was not significantly affected (fig.3), even when the tubulin–myosin ratio was as high as 10:1 w/w.

In another experiment the effect of the column purified calf brain tubulin on the Mg^{2+}-dependent ATPase of rabbit skeletal muscle myosin was examined. This calf brain tubulin from Peak II of the Ultrogel column fractionation (fig.2) also showed no significant effect on the Mg^{2+}-dependent ATPase of rabbit skeletal muscle myosin at weight ratios of tubulin–myosin of between 0.5:1 and 10:1 (table 1).

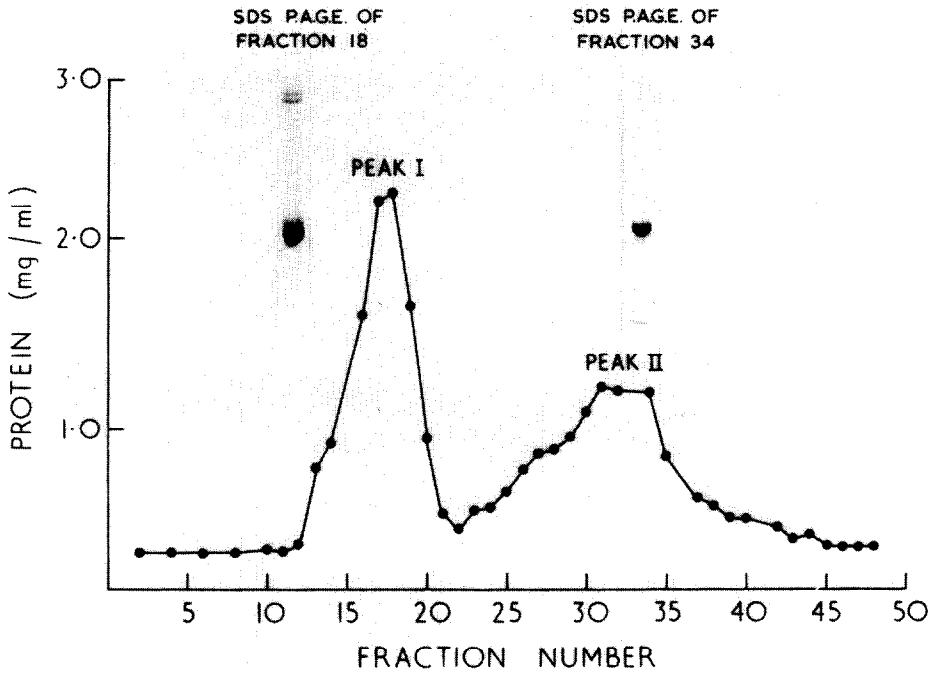

Fig.2. Gel filtration of calf brain microtubule proteins on Ultrogel AcA34. Calf brain microtubule protein was prepared by two cycles of in vitro polymerisation and then depolymerised on ice. The sample in 0.1 M MES, pH 6.8, 1 mM EGTA, 0.1 mM GTP was clarified by centrifugation (100 000 × g_{av} for 1 h at 4°C) and 98 mg of the soluble protein was applied to a 40 × 3.5 cm AcA34 Ultrogel column in a 4°C cold room. The column was previously equilibrated in 0.1 M MES, pH 6.8; 1 mM EGTA; 0.1 mM GTP. Elution was carried out with the same buffer by gravity flow with a flow rate of about 14 ml/h and 70 drop fractions were collected with a LKB 7000 UltroRac Fraction collector. Fractions were assayed for protein by the Lowry procedure (% of recovery of protein from column = 107%) and the peak fractions were further analysed by SDS-polyacrylamide gel electrophoresis (5% gels).

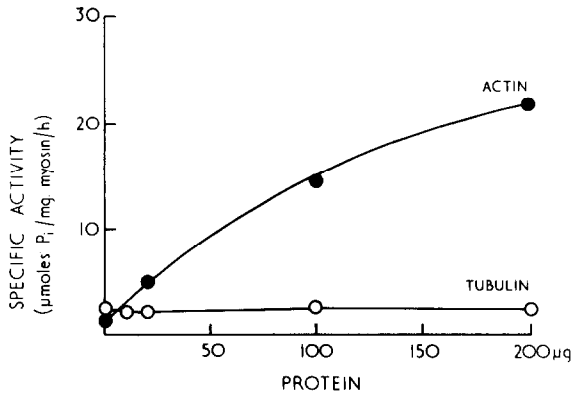

Fig.3. Effect of rabbit skeletal muscle actin and rat brain tubulin on the Mg^{2+}-dependent ATPase activity of rabbit skeletal muscle myosin. Rabbit skeletal muscle actin and rat brain tubulin were prepared by in vitro polymerisation. Increasing amounts (0–200 μg) of actin or tubulin (as indicated) were added to a fixed amount of myosin (20 μg). Each assay mixture contained 30 mM Tris–HCl pH 7.4, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 3 mM Tris–ATP.

Table 1
Effect of column purified bovine brain tubulin on the Mg^{2+}-dependent ATPase or rabbit skeletal muscle myosin

Ratio myosin–tubulin (w/w)	Mg^{2+}-ATPase specific activity[a]
1 : 0	6.38
1 : 0.5	6.51
1 : 1	5.87
1 : 5	6.72
1 : 10	7.81

The bovine brain tubulin used in this experiment was from Peak II of the Ultrogel AcA34 column (see fig.2). Each assay mixture contained 30 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 3 mM Tris ATP.

[a]Activities are expressed as μmol P_i/mg myosin/h.

3.3. *The effect of brain tubulins on the interaction between rabbit skeletal muscle actin and myosin*

The above experiments indicated that adding tubulin alone had no effect on the Mg^{2+}-dependent ATPase of the muscle myosin. It was therefore decided to determine if the presence of tubulin would affect the interaction between actin and myosin. Two experiments were designed to test this possibility. In the first experiment the Mg^{2+}-dependent ATPase activity of rabbit skeletal muscle myosin was stimulated by the addition of increasing amounts of rabbit skeletal muscle actin in the presence and absence of equal amounts (by weight) or rat brain tubulin. The results (table 2) show that the tubulin had little effect on the enzyme activity. In the second experiment, increasing amounts of bovine brain tubulin were added to a myosin–actin mixture (1:1 by weight). Again the presence of tubulin had no significant effect on the observed ATPase activity, even when the ratio of tubulin–actin in the assay mixture was as high as 5:1 w/w (table 3).

3.4. *The effect of varying the pH on the Mg^{2+}-dependent ATPase activity of muscle myosin, measured in the presence and absence of calf brain tubulin*

The effect of the column purified calf brain tubulin (from Peak II, fig.2) on the Mg^{2+}-dependent ATPase of rabbit skeletal muscle myosin was studied throughout the pH range 5.0 to 9.5. A constant weight/weight ratio of myosin–tubulin of 1:1 was used in each assay and the required pH was obtained by using the 'cocktail buffer' described in the Methods section. From the results, (table 4) it can be seen that tubulin

Table 2
Effect of rat brain tubulin on the ability of rabbit skeletal muscle actin to stimulate the Mg^{2+}-dependent ATPase activity of rabbit skeletal muscle myosin

Ratio myosin–actin–tubulin (w/w)	Myosin Mg^{2+}-ATPase specific activity[a]
1:0:0	1.39
1:0.5:0	2.02
1:0.5:0.5	3.09
1:1:0	5.15
1:1:1	5.15

Rabbit skeletal muscle actin and rat brain tubulin were prepared by in vitro polymerisation. A constant amount of rabbit skeletal muscle myosin (20 μg) was used in all the ATPase assays and the actin added in the presence or absence of an equal amount by weight of tubulin. Each assay mixture contained 30 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 3 mM Tris–ATP.

[a]Activities are expressed as μmoles P_i/mg myosin/h.

Table 3
Effect of bovine brain tubulin on the ability of rabbit skeletal muscle actin to stimulate the Mg^{2+}-dependent ATPase activity of rabbit skeletal muscle myosin

Ratio myosin–actin–tubulin (w/w)	Myosin Mg^{2+}-ATPase specific activity[a]
1:0:0	3.30
1:1:0	4.39
1:1:0.25	4.53
1:1:0.5	3.54
1:1:0.75	4.72
1:1:1	4.01
1:1:5	4.72

Rabbit skeletal muscle actin and calf brain tubulin were prepared by in vivo polymerisation. In the assay mixture a constant amount of actin (20 μg) and increasing amounts of tubulin (0–200 μg) were used with 20 μg of myosin. Each assay mixture contained 30 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 3 mM Tris–ATP.

[a]Activities are expressed as μmol P_i/mg myosin/h.

Table 4
Effect of column purified bovine brain tubulin on the Mg^{2+}-dependent ATPase of rabbit skeletal muscle myosin over a pH range of 5.0–9.5

pH	Mg^{2+}-ATPase specific activity[a]	
	Myosin	Myosin + Tubulin
5.0	0	0.08
5.6	0	0
6.0	5.1	5.2
6.6	5.9	5.1
8.5	4.8	5.1
9.5	7.3	6.1

Each assay mixture contained 37 μg of myosin and 37 μg of tubulin and 3 mM $MgCl_2$, 0.1 mM $CaCl_2$ and 3 mM ATP. The total volume of the assay mixture was 2.0 ml of which 1.4 ml was 'cocktail buffer' of the appropriate pH (see methods).

[a]Activities are expressed as μmoles P_i/mg myosin/h.

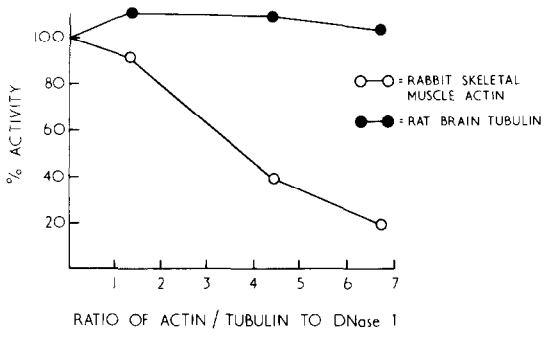

Fig.4. Effect of actin and tubulin on DNase I activity. DNase I activity was measured according to the procedure of Lindberg [19]. Increasing amounts of rabbit skeletal muscle actin (0–33.8 μg) or rat brain tubulin (0–33.8 μg) were added to a fixed amount of DNase I (5 μg) before addition of substrate (120 μg DNA). Each assay mixture (total volume = 3.1 ml) contained 4 mM $MgSO_4$, 1.75 mM $CaCl_2$ and 0.1 M Tris–HCl, pH 7.5.

added in an equivalent amount by weight to the myosin in the assay mixture had no effect on the Mg^{2+}-dependent ATPase activity over the pH range 5.0–9.5.

3.5. *A comparison of the effects of rabbit skeletal muscle actin and mammalian brain tubulins on DNase I activity*

The effects of rabbit skeletal muscle actin and rat brain tubulin on DNase I activity are shown in fig.4. The muscle actin strongly inhibited DNase I activity. At an actin–DNase I ratio of 7:1 (w/w) the enzyme showed only 18% of the activity recorded in the absence of actin. Rat brain tubulin prepared by two cycles of in vitro polymerisation and the column purified calf brain tubulin also had no effect on DNase I activity with tubulin–DNase I ratios of up to 7:1 (w/w).

4. Discussion

In this paper some parallel experiments using skeletal muscle actin and various brain tubulins have been made in an effort to explore possible regions of homology in the structure of the two proteins. With polyacrylamide gel electrophoresis using sodium dodecyl sulphate, the presence of actin as a contaminant in tubulin preparations can be readily detected since rat and calf brain tubulins co-migrate well in this gel system and show mobilities corresponding to a molecular weight of 55 000 ± 2000, whereas rabbit skeletal muscle actin consistently locates ahead of tubulin with an estimated molecular weight 42–44 000. Actin-free rat brain tubulin prepared by two cycles of in vitro polymerisation and calf brain tubulin purified further by gel filtration, both failed to affect the Mg^{2+}-dependent ATPase activity of rabbit skeletal muscle myosin at pH 7.4. Control studies with muscle actin showed that the myosin was not denatured and was capable under low ionic strength conditions of forming synthetic actomyosin complexes with a consequent increase in myosin Mg^{2+}-dependent ATPase activity of around 15-fold. Calf brain tubulin also had no significant effect upon the myosin Mg^{2+}-dependent ATPase activity at any pH within the range 5.0–9.5. In addition, the presence of substantial amounts of tubulin did not appear to affect actin–myosin complex formation since the ATPase activities of actin–myosin mixtures were essentially the same when measured in the presence and absence of brain tubulin.

The effect of actin and tubulin on DNase I activity was also examined and it was shown that whereas rabbit skeletal muscle actin strongly inhibited the DNase I activity, neither rat nor calf tubulin had any significant effect on the enzyme.

These results therefore suggest that actin and tubulin are quite distinct molecular species and do not contain regions of structural homology identifiable by either myosin interaction or DNase I inhibition studies. Furthermore we have shown earlier that actin and tubulin isolated from pig platelets do not appear to have common antigenic determinants [10]. Antibodies raised to tubulin were shown by an immunofluorescence technique to stain quite different structures in cultured bovine lens epithelial cells to the antibodies raised against actin. The antitubulin antibody was shown to react with tubulin but not actin antigen, whereas the anti-actin antibody would react with actin but not tubulin antigen. Moreover, the anti-tubulin fluorescence could be prevented by pre-treatment of the cells with colchicine or exposure to low temperatures, whereas the anti-actin staining was not so affected, but was specifically inhibited by cytochalasin B treatment. If some homology exists therefore between these two proteins it is clearly not well expressed in their

antigenic determinants or in the drug binding sites which affect polymer stability.

Our findings are in broad agreement with those of Mohri and Shimomura [7] but conflict with those Puszkin and Berl [3], Puszkin et al. [4], Camacho and Renaud [20] and Alicea and Renaud [8]. These latter four groups of workers found that tubulins from brain, platelets and the outer doublet fibres of *Tetrahymena* cilia considerably stimulated the Mg^{2+}-dependent ATPase of muscle myosins (for example, as much as 27-fold increases in activity were reported by Puszkin and Berl [3]). It is possible however, that these reports of myosin ATPase stimulation may be accountable for by actin present as a contaminant in their tubulin preparations. In the work of Puszkin and co-workers [3,4] the purity of their protein samples was monitored only by urea polyacrylamide gel electrophoresis and it is known that actin and tubulin have similar migration rates in such systems [21], Furthermore, with certain isolation procedures actin can be a major contaminant of tubulin preparations particularly if cell sources rich in microfilament proteins are used. We have found that crude preparations of platelet tubulin frequently contain a contaminating protein with a molecular weight of about 44 000 on SDS gel (presumptive actin) and that such preparations do slightly stimulate the Mg^{2+}-dependent ATPase activity of muscle myosin (Castle and Crawford, unpublished observations).

Alicea and Renaud [8] found that a preparation of *Tetrahymena* cilia outer fibre tubulin stimulated the chick muscle myosin Mg^{2+}-dependent ATPase about 4-fold with a pH optimum for the stimulation of pH 6.0. Again in their study only urea gels were used to identify the proteins in their preparations and the possibility that some actin perhaps originating from the *Tetrahymena* cell bodies was present in their outer fibre fraction was not explored. Some preliminary results from this laboratory (Marsh 1976, unpublished data) have indicated that *Tetrahymena* outer fibre tubulin prepared by the procedure of Renaud et al. [1] from a cell body-free cilia preparation isolated by the Watson and Hopkins [22] method does not enhance the Mg^{2+}-dependent ATPase activity of rabbit skeletal muscle myosin over a pH range 5.0–8.5.

The possibility that some denaturation of our various tubulin preparations could account for our failure to demonstrate myosin ATPase stimulation can, we think, be discounted since well established procedures have been used and all our preparations were capable of temperature sensitive reversible polymerisation and showed [^{3}H]colchicine binding activities.

In conclusion therefore it is our view that the present results together with the specific fluorescent antibody-labelling studies [10] do not support the concept of actin–tubulin homology at least with respect to their myosin interacting, DNase I inhibiting and drug binding sites and also with respect to their antigenic properties. Tubulin and actin do share some common properties such as similarity in amino acid compositions and their precipitability by Vinca alkaloids. This of course may indicate some phylogenic relationship between the two proteins but further speculation about their structural homology should now await the publication of comparative sequence data.

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